

Challenges in the investigation of the metabolic changes in *Nicotiana attenuata* during insect herbivory using an improved HPLC-TOF-MS method

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Introduction

The target of this study is the investigation of the metabolic changes in leaf extracts of *Nicotiana attenuata* after simulated insect herbivory (see Fig. 1) by mechanically wounding and applying oral secretions of *Manduca sexta* (W+OS) using HPLC-ESI-TOF-MS. Responses of wild-type (WT) plants are primarily compared before investigating the influence of a genetic modification in which the hydroxyproline-rich glycopeptide systemin precursor (ppHypSys) is either overexpressed (OV) or down-regulated (IR). The challenges in this type of study and strategies for quality control are discussed.

Preliminary results obtained from a first data set of the same sample origin (7 time-points with three biological replicates per time-point, treatment and genotype analyzed with a 17min gradient) were used to design the experiment presented here. The number of biological replicates was increased from three to five to access the biological variation. The chromatographic separation was optimized in order to minimize suppression effects due to coelutions. A pooled sample generated from all individual samples was spiked with internal standards (reserpine, atropine) served as quality control sample to monitor instrumental variation.

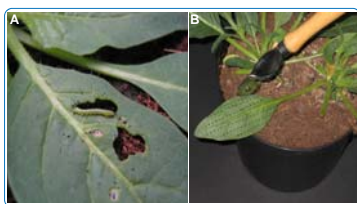


Fig. 1 (A) *Nicotiana attenuata* leaf attacked by *Manduca sexta*. (B) Simulated insect herbivory by wounding leaves with a fabric pattern wheel and applying insect's oral secretions.

Quality Control

The reproducibility of the method was checked by means of the ISTDs reserpine & atropine. The RSD values of peak areas & heights were well below 10% for the entire sequence (Fig. 2A). A pooled sample QC2 representing a 'chemical average' of all samples nicely clusters in the middle of a PCA scores plot of all measured samples. The QC data is used to verify the analytical performance and enables valid interpretation in the complex real samples.

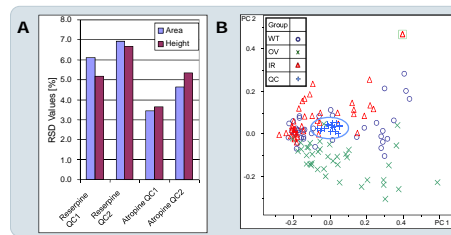


Fig. 2: A- Reproducibility of reserpine & atropine in QC1 (buffer) and QC2 (pooled sample), N=16; B- PCA scores plot of all samples and QC2.

Methods

Treated and untreated leaves (control) from WT, OV, IR (5 biological replicates) were harvested 1, 14, 86, and 120 h after treatment & flash frozen.

Extraction: MeOH/acetic acid buffer 40:60 v/v Internal Standards (ISTD): Atropine, reserpine. QC Samples: QC1: Extraction buffer + ISTD; QC 2: pooled sample of all extracts + ISTD. N= 16 injections.

Eluent A: water + 0.1%AcN+ 0.05% HCOOH
Eluent B: AcN + 0.05% HCOOH
Gradient: 0min-5%B; 2min- 5%B; 2-30min-5-80%B; 35min-80%B; 40min- 5%B; 45min-stop Flow rate: 0.2mL/min

Column: Phenomenex GeminiNX 150x2mm
MS: Bruker micrOTOF & micrOTOF-Q
Ionization: ESI/ positive mode
Scan range: 50-1600 m/z; 1Hz acquisition rate
Calibration: external with NaFormate clusters
Software: ProfileAnalysis for PCA and t-test
Bucketing: time range: 0.5-30min mass range: 50-1600m/z Advanced bucketing; Normalization: None; Scaling: Pareto.

Compounds need to be present in at least 85% of all buckets.

Results

All wildtype (WT) samples were investigated first to identify changes related to time and treatment only using principle component analysis (PCA) and a Student's t-test. The late timepoints of the treated samples are clearly separated in the PCA scores plot (Fig. 3A). The bucket statistics plots for the selected loading at 484.24m/z@11.4min shows the same trend. A t-test of the treated and non-treated WT samples after 120h was applied to narrow down the number of compounds to be investigated for further identification. The t-test result is returned for each bucket with the additional information about the regulation level, the maximum intensity in all analyses and the number of detected compounds per group (Fig. 3B). The first six bucket statistics plot are given as examples (Fig. 3B). In total 59 compounds with p-values < 0.05 and a minimum of 2-fold upregulation in the treated samples were found. This list of compounds was used as the basis for a scheduled precursor list for a targeted AutoMS/MS experiment. Molecular formulae of the target compounds have been generated based on the accurate MS and MS/MS data (Fig. 3C). After statistical analysis, the major challenge is the identification of the compounds involved in regulation. The accurate MS/MS data of the compound 484.24m/z@11.4min has been processed with SmartFormula 3D (Fig. 4A), leading to an MS/MS spectrum assigned with fragment formulae and the corresponding neutral losses (Fig. 4B). With the molecular formula containing 3 nitrogen atoms, a spermidine derivative can be hypothesized. The fragment at 163m/z is typically observed for caffeic acid derivatives, 177m/z for ferulic acid derivatives. The combined information leads to a caffeoyl-feruloyl-spermidine [1] as potential metabolite being upregulated during herbivory. [1] Yohnovsky, N. et. al. Helvetica Chimica Acta 1998 **81**(9) 1654-1671.

Identification

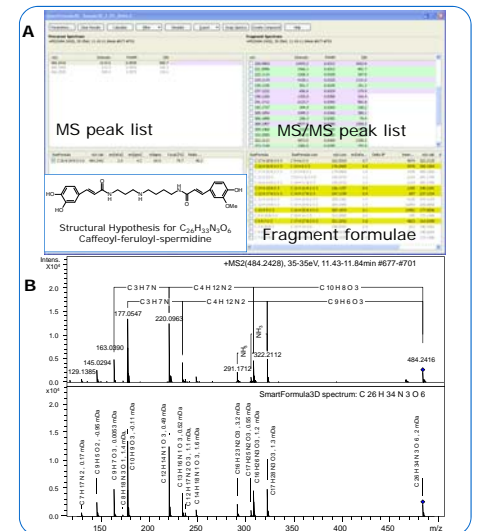


Fig. 4: A- SmartFormula 3D results, B- MS/MS and simulated spectrum with annotated formulae.

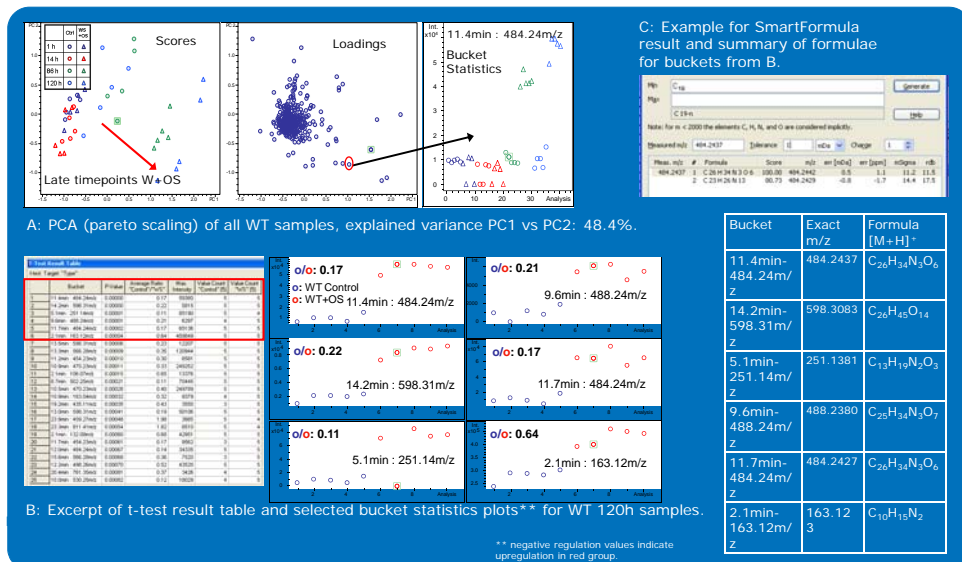


Fig. 3: PCA and t-test results for all wildtype samples and molecular formulae.

Conclusions

- By means of internal standards and a pooled quality control sample, the reproducibility of the analytical method was monitored.
- PCA and t-test were successfully applied to filter out metabolic changes.
- The identification of regulated metabolites can be achieved based on molecular formulae derived from accurate mass spectra plus chemical knowledge.
- The same procedure will be applied to investigate the influence of the genetic modification.